環境バイオテクノロジー学会誌
(Journal of Environmental Biotechnology)
Vol. 1, No. 1, 49－56, 2001

Original paper (regular paper)

Biodegradation of the Sulfonated Azo Dye Direct red 81 by *Shewanella putrefaciens* strain B-3-1 Isolated from River Water

NORIAKI KISHIMOTO[1]*, YOSHIAKI HASHIMOTO[1], TOMOHIRO YOKOBE[1], YOHEI KANAYAMA[1], NORIKO NAKAGAWA[1], SACHIKO YOSHIOKA[1], SADAO KOMEMUSHI[1], AKIRA HIRAISHI[2] AND TOKIO FUJITA[1]

[1]* *Department of Agricultural Chemistry, Faculty of Agriculture, Kinki University, 3327–204 Nakamachi, Nara 631–8505, Japan*
[2] *Department of Ecological Engineering, Toyohashi University of Technology, 1–1 Tenpaku-cho, Toyohashi 441–8580, Japan*
* TEL: 0742–43–1511 (3314) FAX: 0742–43–1445
* E-mail: kisimoto@nara.kindai.ac.jp

(Received 21 September 2001/Accepted 6 December)

An azo dye-degrading bacterium (strain B-3-1) which reduced many different azo dyes was isolated from the Koki River, Osaka, Japan. From the physiological properties and a phylogenetic analysis based on the 16S rDNA sequence of the isolate, strain B-3-1 was classified into *Shewanella putrefaciens*. Under anaerobic conditions, the sulfonated azo dye Direct red 81 was reduced by resting cells of *S. putrefaciens* with lactic acid as an electron donor. Approximately 70% of the Direct red 81 (0.1 mM) added to the reaction mixture was decomposed after 30 min of incubation. The reduction by the resting cells was strongly inhibited by potassium cyanide. Azo reductase activity with NADH as an electron donor was detected in the membrane fraction, but not in the soluble fraction, prepared from cells lysed by an EDTA-lysozyme-Brij 58 protocol. When NADH was used as an electron donor, first cytochrome *c* in the cell membrane was reduced, and then the azo dye was reduced. Potassium cyanide strongly inhibited the reoxidation of reduced cytochrome *c* by Direct red 81, but not the reduction of cytochrome *c* by NADH, suggesting that reduced cytochrome *c* serves as an electron donor for azo dye reduction.

Key words: Shewanella *putrefaciens*, Azo dye, Biodegradation

Azo-dyes are synthetic organic colorants that exhibit great structural diversity[9]. There are currently at least 3,000 azo dyes in use in the textile, paper, and cosmetic industries[13]. Direct and reactive azo dyes in particular are of great commercial importance for the dyeing and printing of cellulose fibers.

Dyes are released into the environment in industrial effluent from two major sources, the textile and the dyestuff industries[10]. They are generally recalcitrant to biodegradation or are incompletely degraded, because aromatic azo and sulfo groups are not synthesized in nature[2,20]. Nevertheless, there have been reports that the azo bond can be reduced by bacteria under anaerobic conditions[3,4].

The most generally accepted hypothesis for this phenomenon is that these strains possess unspecific cytoplasmic enzymes, which act as "flavin-dependent azo reductases" under anaerobic conditions, and transfer electrons via soluble flavins to the azo dyes[24]. However, Russ *et al.*[19] showed that in cell extracts of *Sphingomonas* sp. strain BN6 containing a cloned flavin reductase, the reduction rate for azo compounds increased almost 100-fold, whereas the whole cells exhibited less than 2% of the specific activity obtained with cell extracts. Therefore, they suggested that in vivo the cytoplasmic azo reductases have insignificant importance in the reduction of azo compounds, because the highly polar, sulfonated azo dyes are not able to penetrate the cell membrane. They consequently proposed a different mechanism for the unspecific anaerobic reduction of azo compounds by *Sphingomonas* sp. strain BN6[19]. In this system, the reduction of azo compounds is catalyzed extracellularly by the action of other enzyme systems and/or other redox mediators such as anthraquinone sulfonates.

In this study, from among 369 azo dye-reducing bacteria isolated from rivers and lakes in Osaka and Shiga prefectures, Japan, one strain (designated B-3-1) was selected for

analysis. This strain was identified as *S. putrefaciens* based on physiological properties and phylogenetic analysis, and could reduce different types of azo dyes, including acid, direct, reactive and chrome dyes. The membrane fraction prepared from intact cells could decompose azo compounds by the use of NADH as an electron donor and potassium cyanide completely inhibited this reducing activity. These results suggest that membrane-bound azo reductase and an electron transport component play an important role in the reduction of azo compounds by strain B-3-1. In this article, the first observations of the reduction of an azo dye by *S. putrefaciens* and of the reduction system including electron transport are reported.

Materials and Methods

Microorganisms, media and culture conditions

The bacterium used in this study was selected from among 369 azo dye-reducing bacteria isolated from the Koki and Kashii Rivers, Osaka prefecture, and from Lake Biwa, Shiga prefecture, Japan, in April 1999. The method for the isolation of the azo-reducing bacteria is as follows. Water and mud samples (0.1 ml) from each site were spread over nutrient broth and either malt agar (Nissui Pharmaceutical Co. Ltd.,) or yeast malt extract agar (Difco Lab.,) containing the azo dye Direct red 81 (7-[benzoylamino]-4-hydroxy-3-[[4-[(4-sulfophenyl)azo]phenyl]azo] -2-naphthalene-sulfonic acid, 0.1 g/l, Sigma Chemical Co.), and incubated under aerobic conditions at 28°C for 7 days. A colony that decolorized the azo dye was isolated. The isolate was preserved on a nutrient broth agar slant containing 0.01% Direct red 81 and used throughout this study.

Azo dyes

The following 24 azo dyes, belonging to four different chemical classes, were used in the degradation experiments: (1) acid azo dye group, Acid light red 2bL, Acid yellow 36, Acid yellow 11, Acid black 1; (2) reactive azo dye group, Reactive red 33, Reactive red 120, Reactive red 23, Methyl orange; (3) direct azo dye group, Direct orange 39, Direct yellow 44, Direct yellow 12, Direct orange 26, Direct red 81, Direct blue 78, Direct black 22, Direct black 51; (4) chrome azo dye group, Chrome yellow PG, Chrome green 3B-N, Chrome Black P2B, Chrome Light Gray G, Chrome Bordeaux FB, Chrome yellow 3R, Chrome Black PLW. These dyes were purchased from Miike Dyes Works Ltd. (Omuta, Japan), Nissan Chemical Industries, LTD. (Tokyo, Japan), and Sumitomo Chemical Co., Ltd. (Osaka, Japan).

Identification of the isolated bacterium strain B-3-1

The physiological characteristics of the strain were examined by using API 20E and API 50 CH kits (BioMerieux, France), and compared with the type strain of *Shewanella putrefaciens* IAM 1509^T. Gram stain, sporulation, motility, oxidase and catalase tests were examined in the usual way. The shape of the bacterium was observed by scanning electron microscope (model JEOL LV-5400; JAPAN ELECTRON OPTICS LABO. Co.Ltd.) after the sample was labeled with gold. Quinone compositions of strain B-3-1 and *S. putrefaciens* IAM 1509^T were determined by high-performance liquid chromatography (HPLC)[22].

16S rDNA fragments were amplified by PCR from crude cell lysate and sequenced directly by combined fluorescence detection with a Pharmacia A.L.F. DNA sequencer as described by Hiraishi[5] and Hiraishi *et al.*[6].

Preparation of cell membrane

A membrane fraction was purified from B-3-1 cells using an EDTA-lysozyme-Brij protocol described by Myers and Myers[17]. Cells were cultured aerobically in nutrient broth to the late exponential growth phase and washed with 10 mM Tris-HCl buffer (pH 8.1). The washed cells were suspended in a 25% (wt/wt) sucrose-10 mM Tris-HCl (pH 8.1) solution at 25g (wet weight) of cells per 120 ml of 25% sucrose solution. To accomplish detergent-mediated cell lysis, the following were added with constant stirring at 15-min intervals: 1/10 volume of lysozyme (6.4 mg/ml, Sigma), 1/10 volume of disodium EDTA (20 mg/ml), and 5% (wt/vol) Brij 58 (polyoxyethylene cetyl ether, Sigma) to a final concentration of 0.292% (wt/vol). Cell lysis was confirmed by the decrease of turbidity of cell suspension and observation of cells under microscope. $MgCl_2$ (0.1 M) was then added to a final concentration of 12.8 mM, followed by a few crystals of DNase I (Sigma) to degrade DNA from lytic cell. Cell debris and remaining whole cells were removed by centrifugation at 1,460×g for 15 min. Cell membrane was obtained from these cell extracts by centrifugation for 128 min at 177,500×g in a Hitachi type RP-70T rotor. The resulting pellets were suspended in, and dialyzed against, 50 mM phosphate buffer (pH 7.4). The content of membrane protein was determined by the Lowry method[11].

Anaerobic reduction of azo dye by resting-cell preparations and the membrane fraction

Strain B-3-1 was cultured as described above and suspended to an optical density of 11 at 610 nm in 50 mM phosphate buffer (pH 7.4). The cell suspension was added to a solution (final volume of 2.5 ml in an anaerobic cuvette) containing 100 μmol of phosphate buffer (pH 7.4), 20 μmol of sodium lactate or glucose, distilled water and 0.1 μmol of potassium cyanide, if necessary. The cuvette was sealed hermetically by silicone grease (Dow Corning Co., U.S.A.). Oxygen was removed from the reaction liquid by at least 10 cycles of evacuation and flushing with nitrogen gas (each cycle; 2min). The reaction mixture was preincubated at 28°C for 4 min, and then Direct red 81 (0.25 μmol) was added anaerobically. Direct red 81 (3.378 g l^{-1}) was dissolved in distilled water. The decrease in absorbance at 509 nm was measured spectrophotometrically. Reaction

rates were calculated by using a molar extinction coefficient of 55.2 $mM^{-1}cm^{-1}$. The membrane fraction (120 μg protein) was added to the same solution described above, except that 5 μmol of NADH and 0.025 μmol of FMN were used instead of sodium lactate and glucose.

Absorption spectra and pyridine hemochrome spectrum of the membrane fraction

Reduced and oxidized absorption spectra of the membrane fraction were recorded on a Shimadzu UV-265FS spectrophotometer (Shimadzu Co., Kyoto) on addition of sodium hydrosulfite and aeration, respectively. Samples contained 0.30 mg of membrane protein ml^{-1} in 50 mM phosphate buffer (pH 7.4). The pyridine hemochrome spectrum was determined as described by Myers and Myers[18].

Shift of Soret peak in the membrane fraction on addition of electron donor and azo compound

The membrane fraction (120 μg) was added to a solution (final volume of 2.5 ml in an anaerobic cuvette) containing 125 μmol of phosphate buffer (pH 7.4), 5 μmol of NADH and 0.025 μmol of FMN, distilled water and 0.1 μmol of potassium cyanide, if necessary. After oxygen was removed as described previously, Direct red 81 (0.1 μmol) was added anaerobically. The absorption spectra of each reaction mixture, especially the Soret peaks[7,12] at 418 nm (reduced form) and 409 nm (oxidized form) derived from cytochrome *c*, were recorded.

Identification of the degradation products from Direct red 81

Strain B-3-1 was incubated in 50 mM phosphate buffer (pH 7.4), containing 0.1% (wt/vol) sodium lactate and 0.5% (wt/vol) Direct red 81, at 37°C for 16 h without shaking. Degradation products in the reaction mixture were analyzed by silica gel thin layer chromatography (TLC) using the solvent system chloroform-ethyl acetate-methanol/1 : 1 : 1 (v/v/v) with authentic samples of sulfanilic acid and *p*-phenylenediamine. The spots were detected by ultraviolet irradiation and by spraying 5% phosphomolybdic acid ethanol solution[21].

Results and Discussion

Isolation of azo dye-reducing microorganisms

A total of 369 colonies, comprising 279 bacteria, 81 yeasts and 9 fungi, formed halos on three kinds of agar containing Direct red 81 (Table 1). The number of halo-forming colonies from the Koki and Kashii Rivers was two or three-times that from Lake Biwa. The sampling sites of the Koki and Kashii Rivers were downstream near the river mouth where a few dyehouses have been operating, while the sites at Lake Biwa were lakefront positions of the

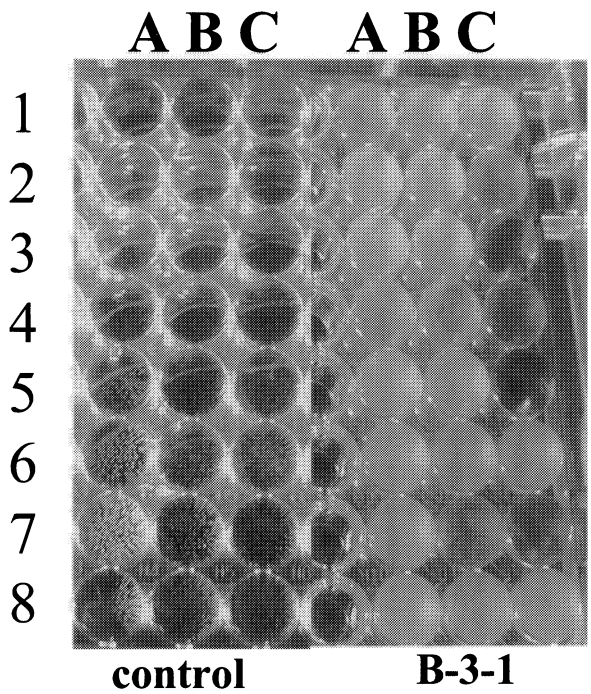

Fig. 1. Decolorization spectra of 24 different azo dyes by strain B-3-1. Column A; lanes 1 to 4 were acid azo dyes; Acid light red 2bL, Acid yellow 36, Acid yellow 11, Acid black 1, and lanes 5 to 8 were reactive azo dyes; Reactive red 33, Reactive red 120, Reactive red 23, Methyl orange. Column B; lanes 1 to 8 were direct azo dyes; Direct orange 39, Direct yellow 44, Direct yellow 12, Direct orange 26, Direct red 81, Direct blue 78, Direct black 22, Direct black 51. Column C; lanes 1 to 7 were chrome azo dyes; Chrome yellow PG, Chrome green 3B-N, Chrome black P2B, Chrome light gray G, Chrome bordeaux FB, Chrome yellow 3R, Chrome black PLW, and lane 8 was Reactive red 22. Strain B-3-1 was cultured in nutrient broth containing 0.05% dye at 28°C for 15h with shaking.

Table 1. Direct red 81-reducing microbes isolated from 5 sites.

	Koki River site 1	Koki River site 2	Kashii River	Lake Biwa site 1	Lake Biwa site 2
Nutrient broth agar	38 (38, 0, 0)	49 (49, 0, 0)	11 (11, 0, 0)	9 (9, 0, 0)	4 (4, 0, 0)
Malt agar	15 (10, 3, 2)	26 (13, 9, 4)	30 (16, 11, 3)	14 (13, 1, 0)	5 (5, 0, 0)
Yeast malt extract agar	30 (17, 13, 0)	58 (37, 21, 0)	48 (30, 18, 0)	23 (18, 5, 0)	9 (9, 0, 0)

The numbers in parentheses indicates the number of colonies of bacteria, yeast, and fungi, respectively. Water and mud samples from each site were spread over agar plates containing azo dye Direct red 81, and incubated at 28°C for 7 days.
The distance between site 1 and 2 was less than 20 m.

southern basin located in a rural area. The greater number of colonies in river water may be attributed to the inflow of industrial effluent from the dyehouse to the river.

Based on the high reducing rate for Direct red 81 and broad degrading spectra against many kinds of azo dyes, strain B-3-1 was selected among 369 colonies. The bacterium was able to completely decolor twenty different azo dyes, both acid, reactive, and direct types, after a 15 h culture in nutrient broth containing 0.05% dye at 28°C (Fig. 1). The microorganism might also partially decompose chrome azo dyes, because after the culture, the color of the culture broth was different from that of the original dye.

From these results, it is thought that strain B-3-1 is suitable for the biodegradation of azo dyes in industrial effluent.

Phylogenetic analysis and physiological characteristics of the azo-reducing bacterium B-3-1

An approximately 1.5 kbp fragment of 16S rDNA from strain B-3-1 was amplified by the polymerase chain reaction, and sequenced directly by the combined method of cycle sequencing and automated fluorescence detection. The sequence has been deposited in the DDBJ nucleotide sequence data base under the accession number AB057660. The sequence of the tested strain was compared with 16S rDNA sequences in the data base, and found to show 98.9% similarity with *Shewanella putrefaciens* LMG 2369.

Strain B-3-1 is a gram-negative, non-spore-forming, facultatively anaerobic and rod-shaped (0.3×1.5 μm) bacterium. The main morphological and biochemical characteristics of the strain were the same as those of *S. putrefaciens* IAM 1509^T (Table 2). Carbohydrates were not utilized as a carbon source for growth, while organic acids, containing lactic and formic acids, were available.

HPLC analysis of a chloroform-methanol extract from strain B-3-1 also indicated peaks at the same retention times (4.46, 5.36, and 6.16 min) as the extract from *S. putrefaciens* IAM 1509^T. The proportion of their peak area from strain B-3-1 (1.0 : 1.1 : 1.7 per r.t. 4.46 : 5.36 : 6.16 min) was similar to that of *S. putrefaciens* IAM 1509^T (1.0 : 1.3 : 2.5). *S. putrefaciens* IAM 1509^T has been reported to contain menaquinone with seven isoprene units and methylmenaquinone with seven isoprene units and ubiquinone with eight isoprene units as the major isoprenoid quinone[1,8]. Strain B-3-1 is presumed to contain the same type of isoprenoid quinone as *S. putrefaciens* IAM 1509^T.

These results show that the isolated strain B-3-1 should be classified as *Shewanella putrefaciens*. To our knowledge, this is the first report on the reduction of azo dyes by *S. putrefaciens*.

Metabolism of Direct red 81 by resting cells of S. putrefaciens B-3-1

Four UV-absorbed spots (Spots a, b, c, and d) were detected on silica gel TLC of the decolored reaction mixture (Fig. 2). Spots c and d coincided in Rf values (0.30 and 0.47) with authentic samples of *p*-phenylenediamine and sulfanilic acid, respectively. These products were isolated by silica gel column chromatography using solvent systems of chloroform-ethyl acetate-methanol / 10 : 7 : 7 and 1 : 1 : 1 (v/v/v). The UV and infrared absorption spectra of the

Table 2. Morphological and physiological characteristics of *Shewanella putrefaciens* IAM 1509^T and strain B-3-1.

	S. putrefaciens IAM 1509^T	B-3-1		*S. putrefaciens* IAM 1509^T	B-3-1
Gram stain	−	−	Acetoin production	−	−
Sporulation	−	−	No_3 reduction	+	+
Form	rod	rod	Denitrification	−	−
Motility	+	+	Acid production from sugar		
Oxidase	+	+	Glucose	−	−
Catalase	+	+	D-Mannitol	−	−
Urease	−	−	Inositol	−	−
β-galactosidase	−	−	D-Sorbitol	−	−
Aginine dihydrolase	−	−	L-Rhamnose	−	−
Ornithine decarboxylase	−	−	Saccharose	−	−
Lysine decarboxylase	−	−	D-Melibiose	−	−
Tryptophan deaminase	+	+	D-Amygdalin	−	−
Utilization of citric acid	−	−	L-Arabinose	+	+
H_2S production	+	+	OF-test	O	O
Indole production	−	−			

^{T} indicates type strain.

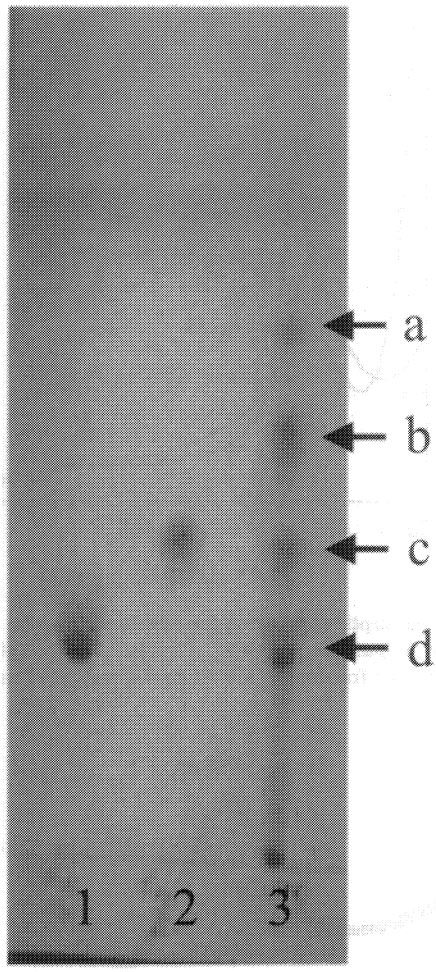

Fig. 2. TLC of reaction products of Direct red 81 reduced by *S. putrefaciens* B-3-1, sulfanilic acid and *p*-phenylenediamine. Lane 1; sulfanilic acid, lane 2; *p*-phenylenediamine, lane 3; reaction products. Plate, silica gel(Merck); Solvent system, chloroform-ethyl acetate-methanol/1 : 1 : 1 (V/V/V). Detection; UV.

isolated products (Spots c and d) were also consistent with authentic *p*-phenylenediamine and sulfanilic acid, respectively.

The initial step in the biodegradation of azo dyes is the reductive cleavage of the azo-bond under anaerobic conditions[14]. Direct red 81 contains two azo-bonds, and therefore, should give three degradation products, sulfanilic acid, *p*-phenylenediamine, and 3-amino-7-benzoylamino-4-hydroxy-naphthalene-2-sulfonic acid. Sulfanilic acid and *p*-phenylenediamine were identifiable by comparing their absorption spectra and Rf values with those of authentic samples. The two residual spots (Spots a and b) in the reaction products are considered further degradation products of 3-amino-7-benzoylamino-4-hydroxy-naphthalene-2-sulfonic acid.

Reduction of Direct red 81 by resting cells of S. putrefaciens B-3-1

Lactic acid caused the reduction of Direct red 81 by resting cells. The azo compound was reduced on addition of 8 mM sodium lactate to the reaction mixture containing resting cells and 0.1 mM azo dye, with 70% of the compound decomposed within 30 min (Fig. 3). Formic acid (8 mM) had an identical rate of decomposition to lactic acid, whereas for 8 mM glucose the rate was one fourth that of lactic acid. Both acids were also available as a carbon source for bacterial growth, and accordingly the reductants among metabolites of these acids might function as electron donors for the cleavage of the azo bond.

The addition of nitrate (5 μmol) to the reaction mixture caused partial inhibition (40%) of the reduction of azo dye by resting cells. Strain B-3-1 has the ability to reduce nitrate (Table 2), and it can therefore be presumed that a part of the reductant formed from lactate metabolism, in the presence of nitrate, is utilized for the reduction of nitrate rather than for the cleavage of the azo group. Myers and Nealson[14] showed that nitrate, Fe(III), and Mn(IV) are used as terminal electron acceptors for anaerobic respiration in *S. putrefaciens*.

The reduction of Direct red 81 by resting cells was completely inhibited by 0.04 mM potassium cyanide, suggesting an involvement of the electron transport system in azo dye reduction.

Reduction of Direct red 81 by the membrane fraction

Under anaerobic conditions, the membrane fraction reduced Direct red 81 on addition of 2 mM NADH as an electron donor, and further addition of 0.01 mM FMN or

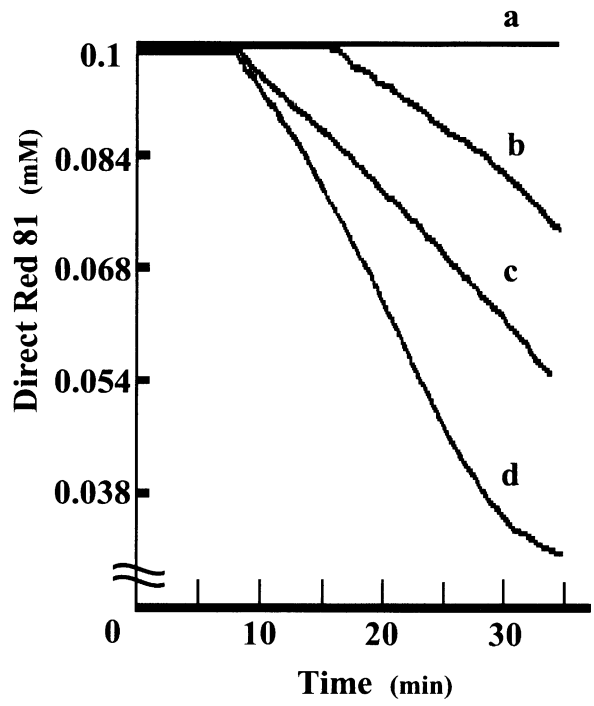

Fig. 3. Reduction of Direct Red 81 by resting cells of *S. putrefaciens* B-3-1 under anaerobic conditions. Curves: a, resting cells+8 mM lactate+0.04 mM KCN+0.1 mM Direct red 81; b, resting cells+8 mM glucose+Direct red 81; c, resting cells+lactate+2 mM NO_3+Direct red 81; d, resting cells+lactate+Direct red 81.

FAD doubled the rate of degradation of azo dye compared with NADH only (Fig. 4). On the other hand, neither 8 mM lactic acid nor 2 mM NADPH was available as an electron donor for azo dye reduction by the membrane fraction. Potassium cyanide (0.04 mM) completely inhibited the reduction of azo dye by the membrane fraction and NADH. In spite of the addition of an electron donor in this study, no activity to reduce azo dye was detected in the soluble fraction. These results suggest that azo dye, in *S. putrefaciens*, is reduced via an electron transport system in the membrane fraction with NADH as an electron donor.

Absorption spectra of the membrane fraction

The reduced and oxidized spectra of the membrane fraction are shown in Fig. 5. The reduced form had a sharp symmetrical α peak at 552 nm, β peak at 523 nm and Soret (γ) peak at 418 nm. The oxidized form had a peak at 409 nm. The pyridine hemochrome spectrum of the membrane fraction had a peak at 550 nm (Fig. 6). These results show the presence of a cytochrome containing heme *c* in the membrane fraction[7,12,18]. A few different *c*-type cytochromes have been purified from *S. putrefaciens*. Morris *et al.*[15] purified flavocytochrome *c* (molecular mass of 63,800 Da) from the periplasmic fraction that contained 4 mol of haem *c* and 1 mol of non-covalently bound FAD per mol of protein. Tsapin *et al.*[23] isolated low-redox-potential

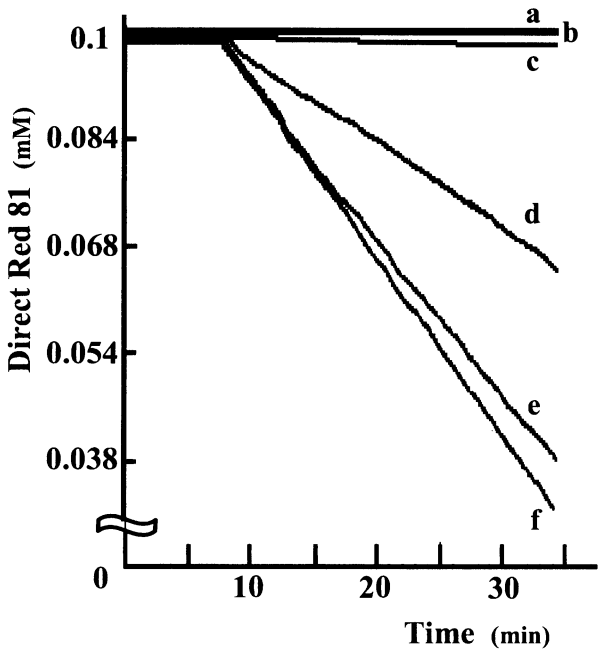

Fig. 4. Reduction of Direct Red 81 by the membrane fraction under anaerobic conditions. Curves: a, membrane fraction+8 mM lactate+0.1 mM Direct red 81; b, membrane fraction+2 mM NADH+0.04 mM KCN+Direct red 81; c, membrane fraction+2 mM NADPH+Direct red 81; d, membrane fraction+NADH+Direct red 81; e, membrane fraction+NADH+0.01 mM FAD+Direct red 81; f, membrane fraction+NADH+0.01 mM FMN+Direct red 81.

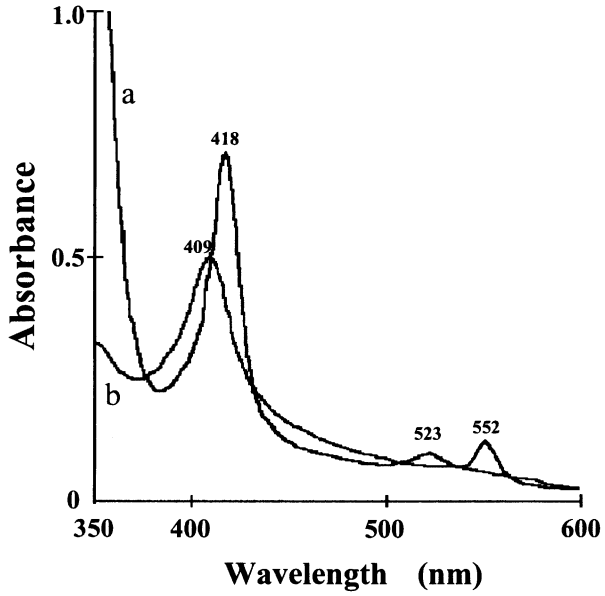

Fig. 5. Absorption spectra of the membrane fraction prepared from *S. putrefaciens* B-3-1. Curves: a, Na2S2O4-reduced membrane fraction; b, air-oxidized membrane fraction.

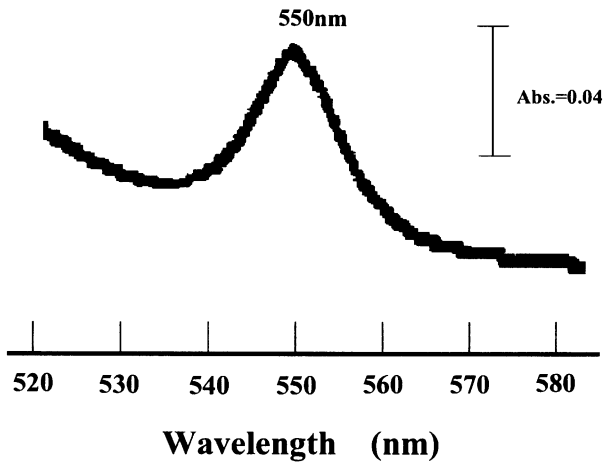

Fig. 6. Reduced spectrum of the pyridine hemochrome of the membrane fraction prepared from *S. putrefaciens* B-3-1.

tetraheme cytochrome *c*3 (molecular mass of 12,120 Da) from the water-soluble fraction of a cell homogenate. Myers and Myers[18] also showed the presence of four distinct outer membrane-bound *c*-type cytochromes (apparent molecular masses of 150, 83, 65, and 53 kDa). The purification of the membrane fraction of strain B-3-1 will reveal which type of cytochrome *c* described above is contained in the membrane.

Shift of Soret peak on addition of electron donor and azo compound

The membrane fraction showed a Soret peak at 409 nm derived from oxidized cytochrome *c* (curve a, Fig. 7), and the addition of 2 mM NADH and 0.01 mM FMN caused the reduction of cytochrome *c* (shift of Soret peak from 409 nm to 418 nm, curve b). Direct red 81 (0.1 mM) reoxidized

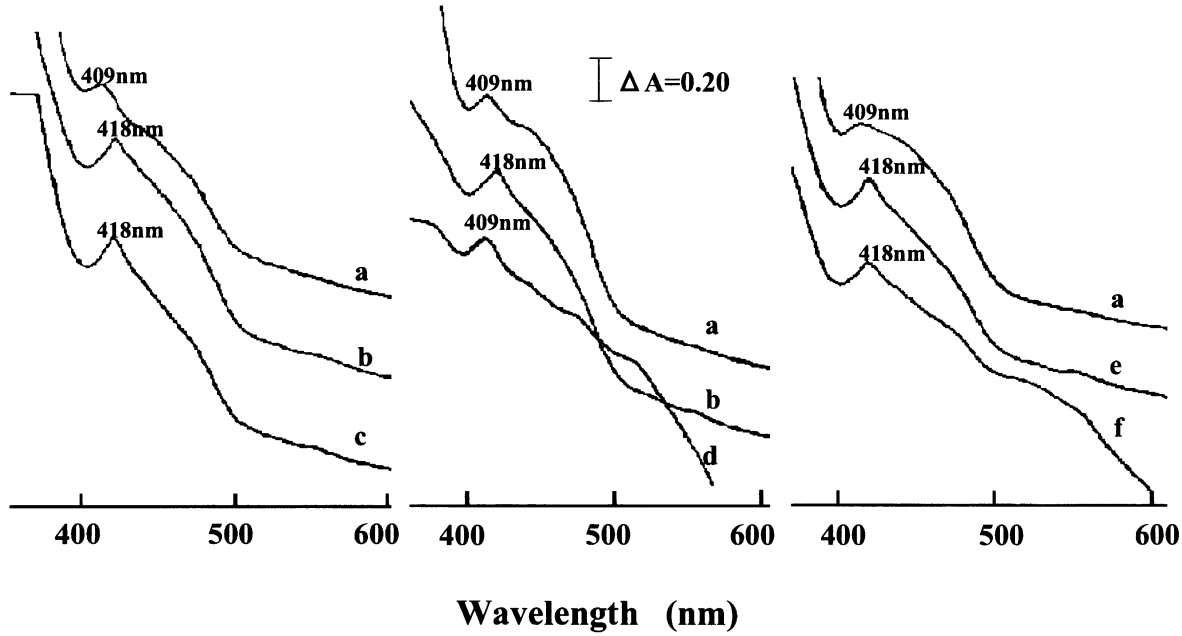

Fig. 7. Shift of Soret peak by the addition of electron donor and azo compound. Curves: a, membrane fraction; b, membrane fraction+2 mM NADH+0.01 mM FMN; c, membrane fraction+NADH+FMN+distilled water; d, membrane fraction+NADH+FMN+0.1 mM Direct red 81; e, membrane fraction+NADH+FMN+0.04 mM KCN; f, membrane fraction+NADH+FMN+KCN+Direct red 81.

the reduced cytochrome *c* (shift of Soret peak from 418 nm to 409 nm, curve d), whereas distilled water was not able to reoxidize it (curve c). Potassium cyanide (0.04 mM) inhibited the reoxidation of reduced cytochrome *c* by Direct red 81 (curve f), but not the reduction of cytochrome *c* by NADH and FMN (curve e). These results show that strain B-3-1 transfers electrons from reductants such as NADH through cytochrome *c* in the membrane to Direct red 81.

Myers and Myers[18] reported that outer membrane cytochromes in the metal-reducing bacterium *S. putrefaciens* functioned as a mediator in the electron transmission to electron acceptors for anaerobic respiration, and that the cytochrome *c* content of the outer membrane markedly increased following a switch from aerobic to anaerobic conditions. We also speculate that under anaerobic conditions, strain B-3-1 reduces azo compounds with the same anaerobic respiration system used for the reduction of oxidized metals and trimethylamine N-oxide. Experiments are currently underway in our lab to purify the membrane-bound cytochrome *c* to clarify its role in the reduction of azo compounds.

References

1) Akagawa-Matsushita, M., T. Itoh, Y. Katayama, and H. Kuraishi. 1992. Isoprenoid quinone composition of some marine *Alteromonas, Marinomonas, Deleya, Pseudomonas* and *Shewanella* species. J. Gen. Microbiol. 138: 2275–2281.
2) Bumpus, J.A. 1995. Microbial degradation of Azo dyes, pp. 157–167. In V.P. Singh(ed.), Microbial degradation of health risk compounds. Elsevier, Amsterdam.
3) Chung, K.T., S.E. Stevens, Jr. and C.E. Cerniglia. 1992. The reduction of azo dyes by the intestinal microflora. Crit. Rev. Microbiol., 18: 175–190.
4) Chung, K.T. and S.E. Stevens, Jr. 1993. Degradation of azo dyes by environmental microorganisms and helminths. Environ. Toxicol. Chem. 12: 2121–2132.
5) Hiraishi, A. 1992. Direct automated sequencing of 16S rDNA amplified by polymerase chain reaction from bacterial cultures without DNA purification. Lett. Appl. Microbiol. 15: 210–213.
6) Hiraishi, A. Shin, Y.K., Ueda, and Y., Sugiyama, J. 1994. Automated sequencing of PCR-amplified 16S rDNA on "Hydrolink" gels. J. Microbio. Methods 19: 145–154.
7) Ingledew, W.J. 1982. *Thiobacillus ferrooxidans* The bioenergetics of an acidophilic chemolithotroph. Biochim. Biophys. Acta 683: 89–117.
8) Itoh, T., H. Funabashi, Y. Katayama-Fujimura, S. Iwasaki, and H. Kuraishi. 1985. Structure of methylmenaquinone-7 isolated from *Alteromonas putrefaciens* IAM 12079. Biochimica et Biophysica Acta 840: 51–55.
9) Johnson, R.F., A. Zenhausern and H. Zollinger. 1978. Azo dyes, pp. 868–910. In H.F. Mark, J.J. McKetta, Jr., D.F. Othmer and A. Standen(ed.), Kirk-Othmer Encyclopedia of Chemical Technology, 2nd ed., Vol. 2. John Wiley & Sons, New York.
10) Leisinger, T. and W. Brunner. 1986. Poorly degradable substances, pp. 497–498. In H.J. Rehm and G. Reed(ed.), Biotechnology, Microbial Degradation, Vol. 8. Verlagsgesellschaft, Weinheim, West Germany.
11) Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.
12) Meyer, T.E., R.G. Bartsch and M.D. Kamen. 1971. A class of electron transfers heme proteins found in both photosynthetic and sulfate-reducing bacteria. Biochim. Biophys. Acta 245: 453–464.
13) Meyer, U. 1981. Biodegradation of synthetic organic colorants. FEMS Symp. 12: 371–385.
14) Meyer, U. 1981. Biodegradation of synthetic organic colorants, pp. 371–385. In T. Leisinger, A.M. Cook, J. Nuesch, and R. Hutter(ed.), Microbial degradation of xenobiotics and recalcitrant compounds, Academic Press, London.

15) Morris, C.J., A.C. Black, S.L. Pealing, F.D.C. Manson, S.K. Chapman, G.A. Reid, D.M. Gibson, and F.B. Ward. 1994. Purification and properties of a novel cytochrome: flavocytochrome c from *Shewanella putrefaciens*. Biochem. J. 302: 587–593.
16) Myers, C.R. and K.H. Nealson. 1990. Respiration-linked proton translocation coupled to anaerobic reduction of manganese(IV) and iron(III) in *Shewanella putrefaciens* MR-1. J. Bacteriol. 172: 6232–6238.
17) Myers, C.R. and J.M. Myers, 1992. Localization of cytochromes to the outer membrane of anaerobically grown *Shewanella putrefaciens* MR-1. J. Bacteriol. 174: 3429–3438.
18) Myers, C.R. and J.M. Myers. 1997. Outer membrane cytochromes of *Shewanella putrefaciens* MR-1: spectral analysis, and purification of the 83-kDa *c*-type cytochrome. Biochim. Biophys. Acta. 1326: 307–318.
19) Russ R., J. Rau, and A. Stolz. 2000. The function of cytoplasmic flavin reductases in the reduction of azo dyes by bacteria. Appl. Environ. Microbiol. 66: 1429–1434.
20) Shaul, G.M. T.J. Holdsworth, C.R. Dempsey, and K.A. Dostall. 1991. Fate of water soluble azo dyes in the activated sludge process. Chemosphere, 22: 107–119.
21) Takitani, S. and M. Suzuki. 1985. Detection on the thin layer chromatography, pp. 74–92. In Method of thin layer chromatography. Kyoritu Publishing Industry, Tokyo.
22) Tamaoka, J. Y. Katayama-Fujimura, and H. Kuraishi. 1983. Analysis of bacterial menaquinone mixtures by high performance liquid chromatography. J. Appl. Microbiol. 54: 31–36.
23) Tsapin, A.I., K.H. Nealson, T. Meyers, M.A. Cusanovich, J.V. Beuumen, L.D. Crosby, B.A. Feinberg, and C. Zhang. 1996. Purification and properties of a low-redox-potential tetraheme cytochrome *c*3 from *Shewanella putrefaciens*. J. Bacteriol. 178: 6386–6388.
24) Walker, R. 1970. The metabolism of azo compounds: a review of the literature. Food Cosmet. Toxicol. 8: 659–676.